Imidazo[1,2-a]Pyridine-3-Carboxamides Are Active Antimicrobial Agents against Mycobacterium avium Infection In Vivo

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Imidazo[1,2-*a*]pyridine-3-carboxamides are Active Antimicrobial Agents of *Mycobacterium avium* Infection *In Vivo*

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Running title: Imidazopyridines as antibiotics against *M. avium*

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Abstract

A panel of six imidazo[1,2-*a*]pyridine-3-carboxamides (IAPs) were shown to have low micromolar activity against *Mycobacterium avium* strains. Compound **ND-10885** (**2**), showed significant activity in the lung, spleen and liver in a mouse *M. avium* infection model. A combined regimen consisting of **ND-10885** (**2**) and rifampin were additive in their anti-*M. avium* activity in the lung. Our data indicates that IAPs represent a new class of antibiotics that are active against *M. avium* and could potentially serve as an effective addition to a combined treatment regimen.

The incidence of non-tuberculosis mycobacteria (NTM) infections has been increasing in the United States (1) (2). *Mycobacterium avium* complex (MAC), which consists of *M. avium* and *M. intracellulare,* is an important cause of both pulmonary disease in individuals with underlying lung diseases such as cystic fibrosis and chronic obstructive pulmonary disease and is an opportunistic pathogen in immunocompromised patients (3, 4). Among the NTM species isolated from U.S. patients, 80% were classified as MAC (5). MAC is ubiquitous within the environment and is found in soil, treated or untreated water, house plumbing systems and animals (6). MAC infection is difficult to treat and has been shown to be resistant to many of the clinically used anti-tuberculosis agents (7, 8). We previously disclosed a novel family of compounds, imidazo[1,2-*a*]pyridine-3-carboxamides (IAPs), with potent activity against *M. tuberculosis* (*Mtb*) (9-12). The mechanism of action and anti-*Mtb in vivo* efficacy of this exciting new class has been documented by us and other groups (9, 13-16). Through a "hit" to "lead" optimization effort aided by the Lilly TB Drug Discovery Initiative (LTBDDI) additional IAP compounds (**1** – **6**) were generated and found to have encouraging *in vivo* pharmacokinetics (PK). Herein, we describe activity of these latest analogs against *M. avium* both *in vitro* and *in vivo.*

Activity of selected compounds *in vitro*. Six compounds having diverse PKs were selected and synthesized following our published methods (9-11). Experimental data and information on all previously uncharacterized compounds (**1, 2** and **6**) can be found in the supporting information section. MIC studies were performed using a resazurin-based colorimetric assay and CFU quantification as described (17). Screening the IAPS against *M. avium* strains 101 and 2151 using standard protocols indicated that they had moderate potency (Table 1). The activity of these compounds against *M. avium* (2.6 – 27.8 µM, two strains) was limited relative to *Mtb* (Table S1) but comparable to positive controls of clarithromycin and azithromycin (1.4 and 13.4 µM, respectively). These compounds also had a good therapeutic window when screened against VERO cells (9) (Table S1).

All six compounds were evaluated for their ability to kill or inhibit *M. avium* replication *in vitro*. Five out of six compounds were bactericidal or bacteriostatic (Fig 2). Consistent with its MIC value, **ND-9903** (**5**) showed no activity against *M. avium* 101 at the highest concentration tested. Separate studies of **ND-9758** (**3**), **ND-9759** (**4**) and **ND-10890** (**6**) at a 1.0 µg/ml each indicated that they were bacteriostatic as the bacterial counts were maintained near the original inoculum. **ND-10885** (**2**) and **ND-9873** (**1**) had bactericidal activity against *M. avium* 101 at 1.0 µg/ml as bacterial numbers decreased by 1 $\log_{10}$ and 0.5 $\log_{10}$, respectively, compared to the original inoculum. Rifampin was the positive control and showed the best bactericidal activity against *M. avium* 101. Except for **ND9903** (**9**), all compounds showed dose-dependent activity against *M. avium*. **ND-10885 (2)** also showed activity against various other *M. avium* clinical isolates of different serotypes, although again it varied 10 fold or more between strains.

Pharmacokinetics. Single-dose pharmacokinetics of compounds **1 - 6** were determined in uninfected 8-week-old male Balb/c mice. Mice received by oral gavage a single dose of compound **1** (at 10 and 100 mg/kg), compound **2** (at 100 mg/kg) , compound **3** (at 10 mg/kg), compound **4** (at 30 mg/kg), compound **5** (at 100 mg/kg), and compound **6** (at 10 and 100 mg/kg). Compounds were analyzed as previously described (10). Calculated parameters include clearance (Cl), area-under-the-curve (AUC), half-life (T1/2), maximum serum concentration (Cmax), time of maximum concentration (Tmax), bioavailability (%F) and percent drug fraction unbound in plasma (Fu, Plasma).

As shown in table 2, all of these compounds had high plasma exposure at their respective doses but there was a large range in half-lives observed (from 2.3 to >24 h). In our drug development paradigm, we selected the maximum free drug concentration per dose (Cmax, u) as the most meaningful pharmacokinetic property to use for compound advancement and **ND-10885** (**2**) and **ND-10890** (**6**) had the highest values (2,795 and 4,232 nM, respectively). The free drug concentration for those two compounds were high enough above their MICs that they were anticipated to be effective against MAC 101 *in vivo*. The PK parameters for compounds **1**, **2** and **6** by IV dose can be found in Table S3.

Maximum tolerated dose. Five compounds were evaluated in mice to determine their maximum tolerated dose as previously described (13). Compounds **ND-9873** (**1**) and **ND-10885** (**2**) were tolerated at the highest concentration of 250 mg/kg for one week, **ND-10890** (**6**) was well-tolerated at 100 mg/kg for one week and as previously reported **ND-9759** (**5**) was tolerated at 30 mg/kg for 28 days (13). Mice treated with **ND-9758** (**3**) did not tolerate the drug even at the lowest concentration (30 mg/kg).

Efficacy of ND10885 in MAC-infected mice. We chose compound **ND-10885** (**2**) for the *in vivo* studies in Wild type Balb/c mice based on its relatively good *in vitro* bactericidal activity against *M. avium,* its PK profile and its low toxicity in mice. Wild type Balb/c mice (n=3) were retro-orbitally infected with *M. avium* MAC 101 at a dose of 10^7 CFU in 50 µL of PBS (18). One week after the infection, mice were treated by oral gavage with **ND-10885** (**2**) dissolved in 80% propylene glycol (v/v) once daily 6 days a week for two weeks. After the final dosing, all mice were sacrificed, and the mycobacaterial burden was determined as described previously (13). The bacterial numbers were quantified by visually counting bacterial colonies. At the beginning of treatment, a group of mice (n=3) were sacrificed to measure the mycobacterial input in the lung, spleen and liver. As a negative control, a group of mice (n=3) were treated with the vehicle, 80% propylene glycol, only.

ND-10885 (**2**) significantly inhibited *M. avium* growth in the lungs, spleens and livers when compared to the vehicle-treated *M. avium*-infected mice (Fig 3). The inhibitory activity of **ND-10885** (**2**) was comparable to that of rifampin in all three organs. In addition, compared to single compound/drug regimens, mycobacterial counts were lower in all three organs when *M. avium*-infected mice were treated with a combined regimen of **ND-10885** (**2**) and rifampin, although this was only statistically significant in the lung. When compared with baseline mycobacterial counts at the initiation of treatment, the combined regimen showed bactericidal activity with the CFU count decreasing 1.5-fold (Log_{10}) in the lung.

In conclusion, we show that IAPs are active against *M. avium* clinical isolates. One compound**, ND-10885** (**2**), has significant activity against *M. avium* in mice, reducing bacterial burden in the lung, spleen and liver when compared to the untreated group. Most interestingly, **ND-10885** (**2**) has activity comparable to rifampin, a critical component in the combined regimen used to treat MAC infections (8) and a combined regimen consisting of **ND-10885** (**2**) and rifampin showed enhanced bactericidal activity in the lung. Our data suggest that IAPs should be pursued as a new class of compounds to treat *M. avium* and perhaps other NTMs.

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Mulugeta S, **Franzblau SG**, **Miller MJ**. 2015. Putting Tuberculosis (TB) To Rest: Transformation of the Sleep Aid, Ambien, and "Anagrams" Generated Potent Antituberculosis Agents. ACS Infect Dis **1**:85–90.

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Med **19**:1157–1160.

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191 Table 1. Screening of compounds **1 – 6** against two strains of *M. avium*. MICs are shown in µM.

Compound	Mol. Wt.	Clog P	MAC 101 (serotype 1)	MAC 2151 (serotype 2)
ND-9873 (1)	363.34	4.6	2.8	27.5
ND-10885 (2)	321.38	3.6	1.6	15.6
ND-9758 (3)	389.43	5.8	1.28	2.57
ND-9759 (4)	405.88	6.4	0.31	1.2
ND-9903 (5)	425.41	6.4	>23.5	>23.5
ND-10890 (6)	382.44	3.4	2.6	13.1
Rifampin	822.95	6.04	0.077	0.158
Ethambutol	204.31	0.12	48.9	>48.9
Clarithromycin	747.95	2.82	1.4	1.4
Azithromycin	748.88	2.28	13.4	>13.4

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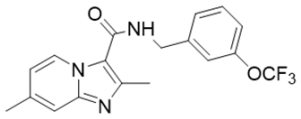
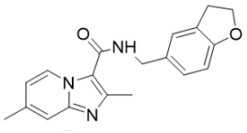
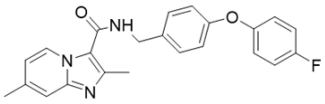
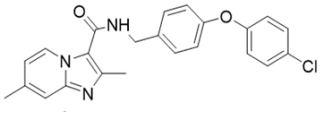
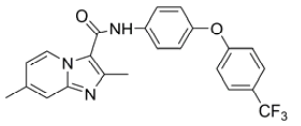
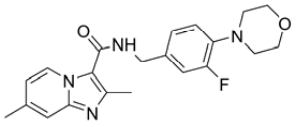
196 Table 2. Pharmacokinetic parameters of compounds **1 – 6**.

	***in vivo* PK**	
Compound	**PK Value**	**PK calculation**
 **ND-9873 (1)**	AUC(ng*Hours/mL) =27,768 (PO; 10 mg/kg) T1/2 (Hrs)=2.91 F%=51 Cmax (ng/mL)= 10,294 Tmax (Hrs)=1.0 Cl (mL/Min./Kg)=20 Fu, PLasma= 0.023	- Cmax, u (100 mg/kg, PO) = 652 nM
 **ND-10885 (2)**	AUC(ng*Hours/mL) =51,249.7 (PO; 100 mg/kg) T1/2 (Hrs)=2.35 F%=44.3 Cmax (ng/mL)= 19,530 Tmax (Hrs)=0.333 Cl (mL/Min./Kg)=4.25 Fu, PLasma= 0.046	- Cmax, u (100 mg/kg, PO) = 2,795 nM
 **ND-9758 (3)**	AUC(ng*Hours/mL) =11,000 (PO; 10 mg/kg) T1/2 (Hrs)=13.2 F%=ND Cmax (ng/mL)= 1,160 Tmax (Hrs)=0.50 Cl (mL/Min./Kg)=NDA Fu, PLasma= 0.003	ND
 **ND-9759 (4)**	AUC(ng*Hours/mL) =22,200 (PO; 30 mg/kg) AUC T1/2 (Hrs) = 20.2 F% = ND Cmax (ng/mL)= 2,900 Tmax (Hrs) = 1.00 Cl (mL/Min./Kg)=NDA Fu, PLasma=0.001	- Cmax, u (30 mg/kg, PO) = 7 nM
 **ND-9903 (5)**	AUC(ng*Hours/mL) =594,000 (PO; 100 mg/kg) T1/2 (Hrs) > 24 F% = ND Cmax (ng/mL)= 34,900 Tmax (Hrs)=12 Cl (mL/Min./Kg)=NDA Fu, PLasma=0.0016	Cmax, u (100 mg/kg, PO) = 133 nM
 **ND-10890 (6)**	AUC(ng*Hours/mL) =32,800 (PO; 10 mg/kg) T1/2 (Hrs)=3.96 F%=46.3 Cmax (ng/mL)= 11,500 Tmax (Hrs)=0.250 Cl (mL/Min./Kg)=2.32 Fu, PLasma=0.039	- Cmax, u (10 mg/kg, PO) = 1,232 nM - Cmax, u (100 mg/kg,PO) = 4,232 nM

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198 **ND; not determined**

Figure Legends

Fig. 1. Anti-*M. avium* activities of Imidazo[1,2-*a*]pyridine-3-carboxyamides *in vitro* measured by CFU counts. *M. avium* were treated with compounds at various concentrations as shown, and then bacterial CFU were determined on Middlebrook 7H10 agar plates. Baseline, *M. avium* CFU at the beginning of treatment. The results are representatives of three independent experiments. *, $p < 0.05$ compared to the Baseline (one-way ANOVA with Tukey post-test).

Fig. 2. Efficacy of ND10885 (6) against *M. avium* infection in the Balb/c mouse model. *M. avium*-infected mice were treated with compounds once daily, 6 day per week for two weeks. Bacterial burden in the lung, spleen and liver was determined. Mock, mice treated with vehicle alone. Baseline, bacterial burden at the beginning of treatment. The results are representatives of two independent experiments. *, $p < 0.05$ compared to the mock, ** $p < 0.05$ compared to ND10885 (100 mg/kg) (one-way ANOVA with Tukey post-test).

Fig. 1

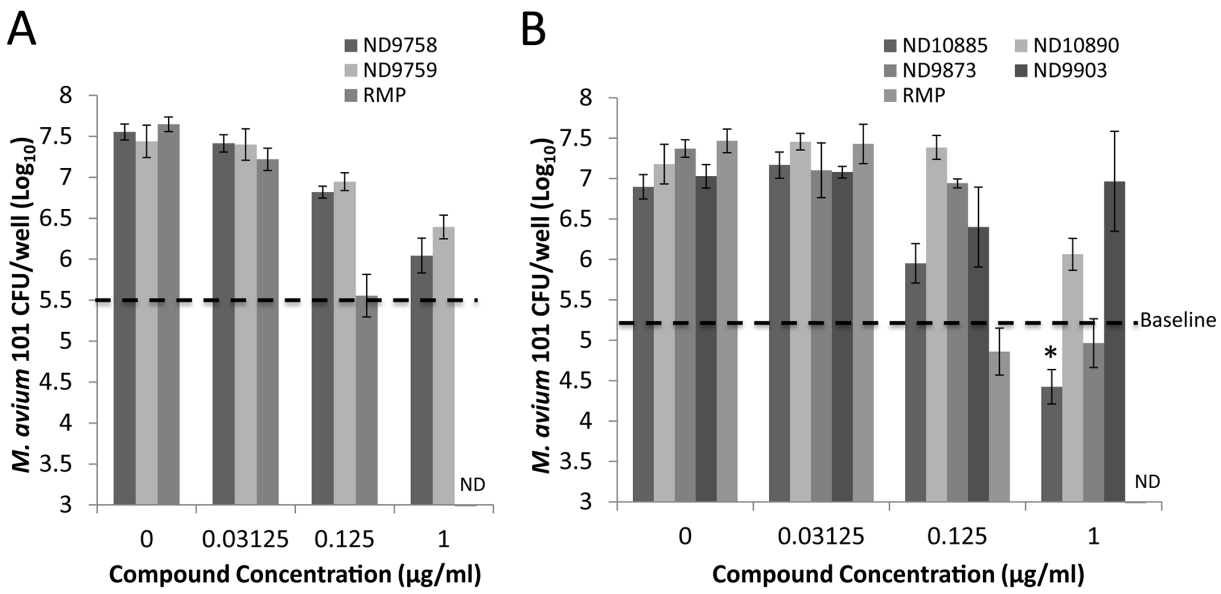

Fig. 2